of free NADH indicated by a distribution shifted more toward the shorter lifetime as compared to the phasor distribution of the cytoplasm. Also within the nucleus, two distinct lifetime distributions were observed with the perinuclear region having more bound fraction of NADH and an abundance of free NADH in the central nucleolus region. A similar observation was reported in myoblasts [Microscopy research and technique,75(12),1717-22,(2012)]. Furthermore, within the cytoplasm there are some regions with more bound NADH. This could be due to presence of more protein bound NADH within the mitochondria. The phasor analysis also revealed a unique long lifetime distribution which arose from small granular like structures within the iPS cells. These areas also had higher intensities compared to rest of the cells. This could be liquid droplet-associated granules (LDAGs) which were previously observed by Stringari et al [Journal of biomedical optics 17(4),046012,(2012)] in embryonic stem cell. Hence, we show mapping of metabolic activity of iPS cells at subcellular resolution employing a non-invasive, label-free, optical microscopy technique.

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Pulsed Interleaved Excitation Fluctuation Imaging: Method and Application to HIV-1 Assembly

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Pulsed interleaved excitation (PIE) is the method of nanosecond alternating excitation with time-resolved detection and allows accurate, independent and quasi-simultaneous determination of fluorescence intensities and lifetimes of different fluorophores. We have combined PIE with fluctuation imaging methods (PIE-FI) such as raster image correlation spectroscopy (RICS) and number and brightness analysis (N&B) on a home-built multicolor laser/sample scanning microscope. PIE-FI is a novel method that allows robust and accurate measurements of concentrations (down to pM), diffusion and stoichiometry, crosstalk-free dual-color measurements to probe protein-protein interactions, dual-color fluorescence lifetime measurements to quantify Förster resonance energy transfer and even allows resolving different species in images on the basis of their fluorescence lifetime rather than their color. We have applied PIE-FI to study the HIV-1 Gag protein inside live cells. During de novo synthesis of HIV particles in infected cells, this protein assembles at the plasma membrane and creates the shell that ultimately defines the size and shape of budded virions. Little is known about interactions of HIV Gag with cellular and/or viral components inside the cytosol of infected cells. We investigated the wild-type HIV Gag as well as some critical site-specific mutants that influence membrane and/or RNA and/or auto-interactions of Gag to gain more insight into these early stages of HIV assembly.

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Live-Cell Super-Resolution Imaging of Endogenous Ligand-Activated Protein Dimers by Combining Upaint and Single Molecule FRET

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Molecular interactions are key to many chemical and biological processes like protein function. In many signaling processes they occur in subcellular areas displaying nanoscale organizations and involving molecular assemblies. The nanometric dimensions and the dynamic nature of the interactions make their investigations complex in live cells. While super-resolution fluorescence microscopies offer live-cell molecular imaging with sub-wavelength resolutions, they lack specificity for distinguishing interacting molecule populations. Here we combine the uPAINT super-resolution microscopy method (1) and single-molecule Förster Resonance Energy Transfer (FRET) to identify dimers of endogenous receptors induced by ligand binding and provide super-resolved images of their membrane distribution in live cells (2). By developing a two-color universal-Point-Accumulation-In-the-Nanoscale-Topography (uPAINT) method, dimers of epidermal growth factor receptors (EGFR) activated by EGF are studied at ultra-high densities, revealing preferential cell-edge sub-localization (3). We will also discuss the versatility of uPAINT when using different fluorescent ligand systems (antibodies, activating ligands, synthetic ligands etc...) for targeting and imaging different types of membrane proteins with superresolution, including endogenous receptors (1-3).

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Development of Pump-Probe Nanoscopy Architecture

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Although modern optical microscopy allows the achievement of sub-diffraction resolution, most of the current techniques rely on a fluorescence contrast mechanism. Moreover, deep tissue imaging remains a challenging task especially for thick and highly scattering biological objects. The infrared absorption/saturation microscopy method is designed to overcome these issues [1, 2], having InfraRed Nanoscopy (IRN) as an instrumental perspective. The main idea behind IRN is an absorption/saturation effect similar to conventional pump-probe in which the first pump beam modifies the carrier density inside the sample, followed by intensity changes in the transmitted probe beam. By introducing an additional doughnut shaped pump beam one can transiently saturate the induced transition in the periphery of the focal spot, and collect the signal generated from the central sub-diffraction area. This is useful mainly for infrared absorption microscopy. The IR range operates in the transparency window of biological tissues and by selecting specific wavelengths one can observe selected vibrational modes. Therefore, such a technique could be applied for the imaging of non-fluorescent species, which is important for various biological applications.

In this work we outline the architecture of a saturated transient absorption microscope, directed towards the development of a microscopy platform comprising a pump-probe setup and STED nanoscope [3]. Pump and probe wavelengths can be tuned from the visible (400-500 nm) to the near IR (800-1000 nm) spectral ranges, according to the sample properties. This permits accessing explicit spatial and dynamical information about the sample for applications in cellular biophysics and nanochemistry. We also indicate the possibility of implementing 3D super-resolution capability.

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Super-Resolution Imaging and Single Molecule Tracking of the Nuclear Protein Emerin

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Emerin is an integral protein of the nuclear membrane expressed in most somatic cells. When under-expressed or mutated, it leads to Emery-Dreifuss Muscular Dystrophy. This disease is characterized by cardiac abnormalities and progressive muscle wasting. The molecular bases of these phenotypes are not well defined. Previous attempts at characterizing emerin localization and diffusional mobility have relied on ensemble confocal fluorescence microscopy. This approach did not reveal differences between normal and mutated versions of emerin, perhaps due to the limited optical resolution of confocal and FRAP techniques. Here we employ single molecule tracking and super-resolution optical imaging of emerin to further characterize emerin diffusion behaviors and localization at the nanometer scale.

We performed single protein tracking of photoactivable-TagRFP-emerin fusions in live cells and study their dynamics in the nuclear membrane. Diffusion analyses indicate the co-existence of four diffusing sub-populations. One emerin sub-population is associated with the endoplasmic reticulum diffusion, two are located exclusively to the nuclear membrane, and a nearly immobile sub-population also locates to the nuclear membrane. Single molecule pulse-chase experiments reveal that the two nuclear-exclusive sub-populations accumulate at different rates, providing further evidence of their mutual uniqueness and their differential distribution between the inner and outer nuclear membrane. Further studies with disease-associated emerin mutants revealed that a single point emerin mutant has a 250% increase in diffusion coefficient for one of the nuclear membrane sub-populations when compared to the wild type.

We also map the 3D nanoscale organization of emerin at the nuclear membrane using super-resolution imaging of SNAP-tag-emerin fusions. Analysis of emerin clusters was performed with 3D Ripley's K-function to compare the distribution of wild-type and mutated versions of emerin.